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Partial Purification and Demonstration of Anti-tumor Activity of High Molecular Weight DNA-binding Protein (HMDBP) in Ascitic Fluid of Mice with Meth-A Ascites Fibrosarcoma

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INTRODUCTION

A number of DNA-binding proteins have been separated from the cytoplasm[1], serum[2~6] and cerebrospinal fluid (7) of a variety of animals. Since some of these DNA-binding proteins are increased in animals bearing malignant tumors[3~5] it has been suggested that determining their serum levels might aid in the diagnosis of cancer. Recently Krøll et al.[8] reported that DNA-binding proteins from Yoshida ascitic fluid can suppress the growth of tumor cells, and other authors reported that high molecular weight proteins in the serum (nHG-200)[9] and cultured media (160,000)[10] can suppress tumor cell growth in vitro. These observations seem to indicate that high molecular weight DNA-binding proteins can suppress the growth of tumor cells in Yoshida ascitic fluid[8]. Our present experiments show that high molecular weight DNA-binding proteins in the ascitic fluid of mice with Meth-A fibrosarcoma can also inhibit tumor growth. The main protein in this group has a molecular weight of 160,000 daltons.

MATERIALS AND METHODS

Ascites tumor Meth-A ascites fibrosarcoma, serially transplanted in BALB/c mice was kindly contributed by Dr. K. Kawashima (First Dept. of Internal Medicine, Nagoya University School of Medicine) and Dr. S. Muramatsu (Faculty of Science, Kyoto University) and transplanted into syngenic mice in our laboratory.

DNA-affinity chromatography DNA-cellulose was prepared by coupling native calf thymus DNA (type 1) (Sigma Chemical Co., Mo) to Whatman CF 11 cellulose particles by the method of Alberts and Herrick[11]. DNA-binding proteins were prepared by the method of Parsons et al.[4]. All steps of the procedure were performed at 4°C, and all buffers contained 1 mM EDTA and 1 mM 2-mercaptoethanol. The proteins adsorbed on the DNA-cellulose column were eluted with 10 mM phosphate buffer (pH 6.8) containing 0.5 M NaCl.

Partial purification of HMDBP The DNA-binding proteins thus obtained were applied to a DEAE-cellulose (DE 52) column equilibrated with 10 mM phosphate buffer (pH 6.8). The

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column was washed with the same buffer solution until no further protein could be detected in the eluate. Elution was performed with 10 mM phosphate buffer (pH 6.8) containing 0.12 M KCl, and the eluates obtained were dialyzed against distilled water and lyophilized. DNA-binding proteins thus purified were subjected to isoelectric focusing chromatography carried out under pH gradients between 5.0 and 8.0. A subfraction fractionated at pH 4.8 was collected and developed on Sephadex G-25 column chromatography. The protein thus obtained was rechromatographed on a Sephadex G-200 column (3×90 cm) equilibrated with 20 mM phosphate buffer (pH 7.2) containing 0.14 M KCl. The first peak was pooled as HMDBP.

Calculation of molecular weight of HMDBP Sodium dodecyl-sulfate polyacrylamide gel electrophoresis was employed for calculating the molecular weights of proteins by the method of Weber and Osborn[12]. Heat resistant RNA polymerase B proteins (Seikagaku Kogyo Co., Ltd., Tokyo) were used as markers.

Immunoelectrophoresis and Ouchterlony test Immunoelectrophoresis was performed on 1.0% agarose with 0.1 M veronal buffer (pH 6.8) containing 2 mM calcium lactate. The Ouchterlony test was performed in 1.0% agarose with PBS (pH 7.2). Rabbit antisera against mouse immunoglobulins, IgA, IgM and IgG, and against whole mouse serum were purchased from MBL, Ltd., Nagoya. Antiserum against 160,000 dalton protein was produced in rabbits in our laboratory, and the antigen used for immunization was separated from sodium dodecylsulfate polyacrylamide gel electrophoresis by the method of Weintraub et al.[13].

Amount of protein The amount of protein was determined by the method of Lowry et al.[14].

Inhibition assay of tumor cell growth in vitro Assays were carried out in a low concentration of fetal calf serum (FCS) by the method of Holly et al.[15]. These conditions are very useful in assaying cell growth inhibition, because small quantites of samples can be tested. The conditions employed were different from their FCS concentrations. Meth-A fibrosarcoma cells were suitable to grow in 0.3% rather than 0.1% FCS. Meth-A fibrosarcoma (3×10^5 cells) was cultured in Dulbecco's modified Eagle medium (DEM) containing 5% FCS in a 60 mm Petri dish. A xenogeneic cell line, HeLa S3, was cultured in DEM containing 10% FCS. Twenty four hours after the initiation of these cultures, the medium was replaced by DEM containing 0.3% FCS and cultured for 48 hrs. Highly purified HMDBP diluted with DEM to a final concentration of 10 μg/ml was added to the culture at the time of the medium change. For examining ^{3}H-thymidine incorporation, 1 μCi of ^{3}H-thymidine (New England Nuclear, Boston, Mass.) dissolved in PBS (pH 7.2) to a final concentration of 10 μCi/ml was added to each dish when the medium was changed. The cultured cells were washed with cold PBS (pH 7.2) three times at the termination of culture in order to remove free ^{3}H-thymidine. The cellular material was dissolved in NaOH at 56°C, and DNA fractions were precipitated by 10% trichloroacetic acid. The precipitates were dissolved in 28% ammonium hydroxide, mixed with 10 ml of Univer-gel (Nakarai Chemicals, Ltd., Kyoto), kept overnight, and the radioactivity was counted in a Packard Tri-Carb liquid scintillation counter. In addition to the radioactivity measurement, the number of cultured cells at the initiation and the termination of culture was calculated with a Coulter counter. These experiments were carried out in duplicate.

RESULTS

Partial purification and characteristics of HMDBP The DNA-binding proteins obtained from mouse ascites tumor fluid were separated on disc-gel electrophoresis in 5% polyacrylamide gel. Fig. 1 shows the patterns obtained when the blocks were stained with Coomassie brilliant blue. Fifteen or more protein fraction were demonstrated in the first disc in which the DNA-binding protein fraction was separated. The major high molecular weight proteins were calculated to be 160,000 and 200,000 or more daltons in molecular weight (Fig. 1–a). All DNA-binding proteins were stainable with PAS, so were glycoproteins. To isolate HMDBP of 160,000 daltons, the

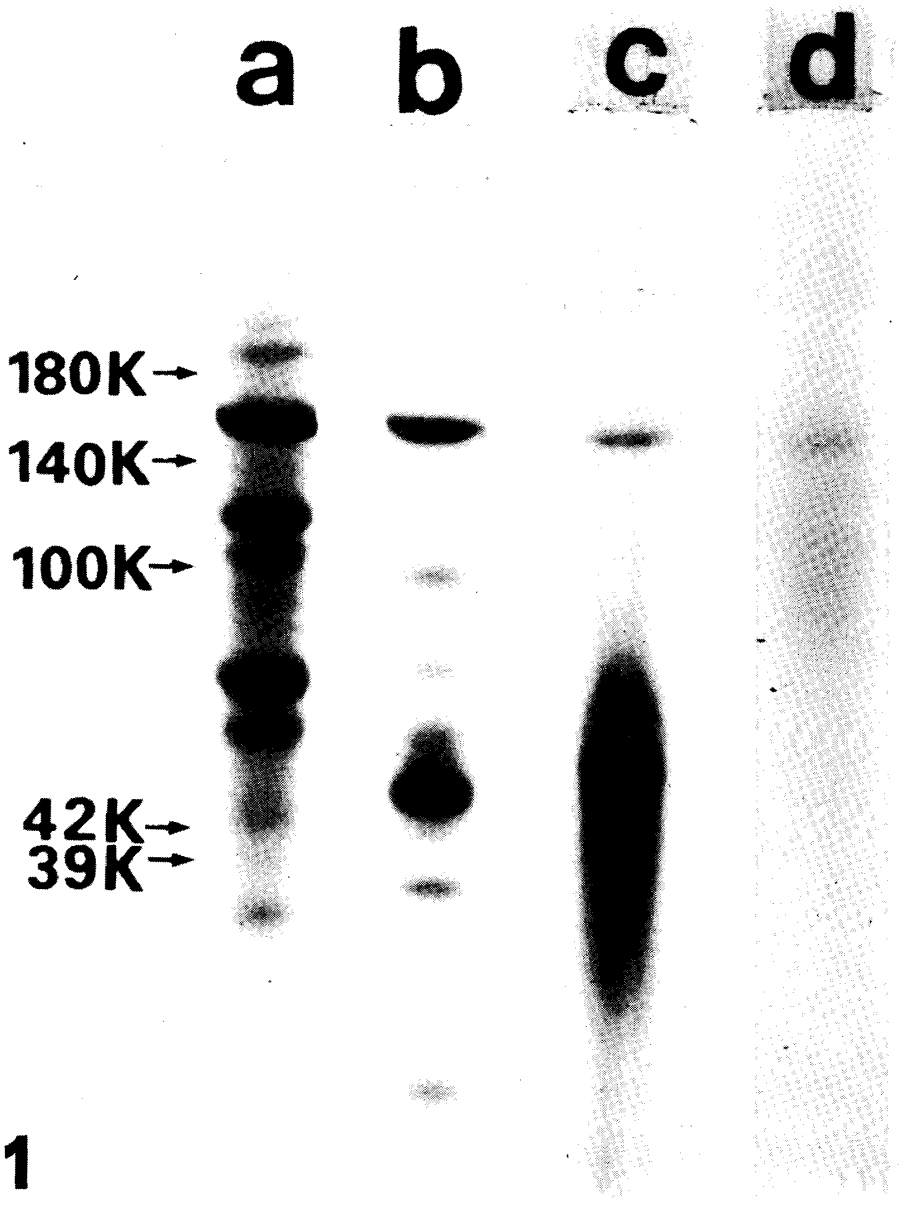

Fig. 1. Steps in purification of HMDBP from DNA-binding proteins on 5% sodium dodecylsulfate polyacrylamide gel electrophoresis. Each sample was dissolved in 50 mM phosphate buffer (pH 7.2) containing 5% SDS and 2% 2-mercaptoethanol and boiled in a water bath at 90°C for 90 seconds. (a): DNA-binding proteins in ascitic fluid, (b): 0.12 M KCl eluates from DNA-binding proteins on DEAE-cellulose, (c): pH 4.8 fraction in isoelectric focusing, (d): partially purified HMDBP from Sephadex G-200 gel chromatography.

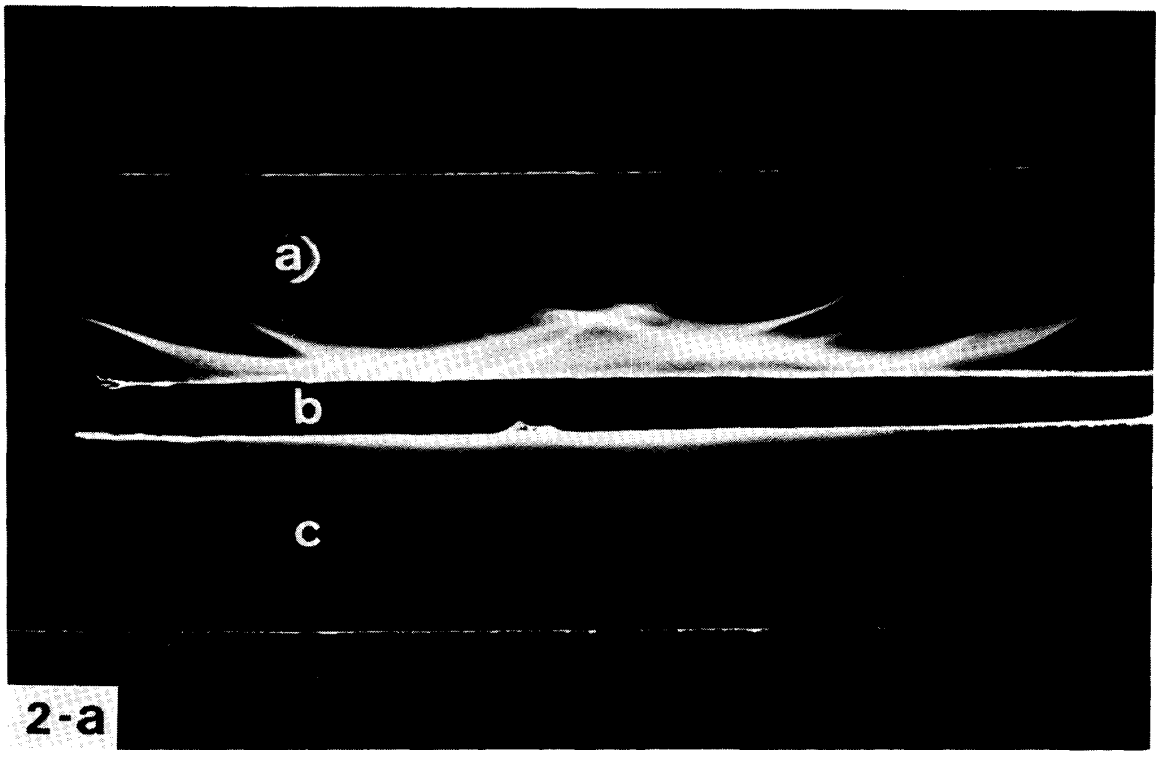

Fig. 2-(a). Immunoelectrophoretic pattern of HMDBP. (a): 200 μg of normal mouse serum, (b): rabbit anti-mouse serum anti-serum, (c): 10 μg of partially purified HMDBP.

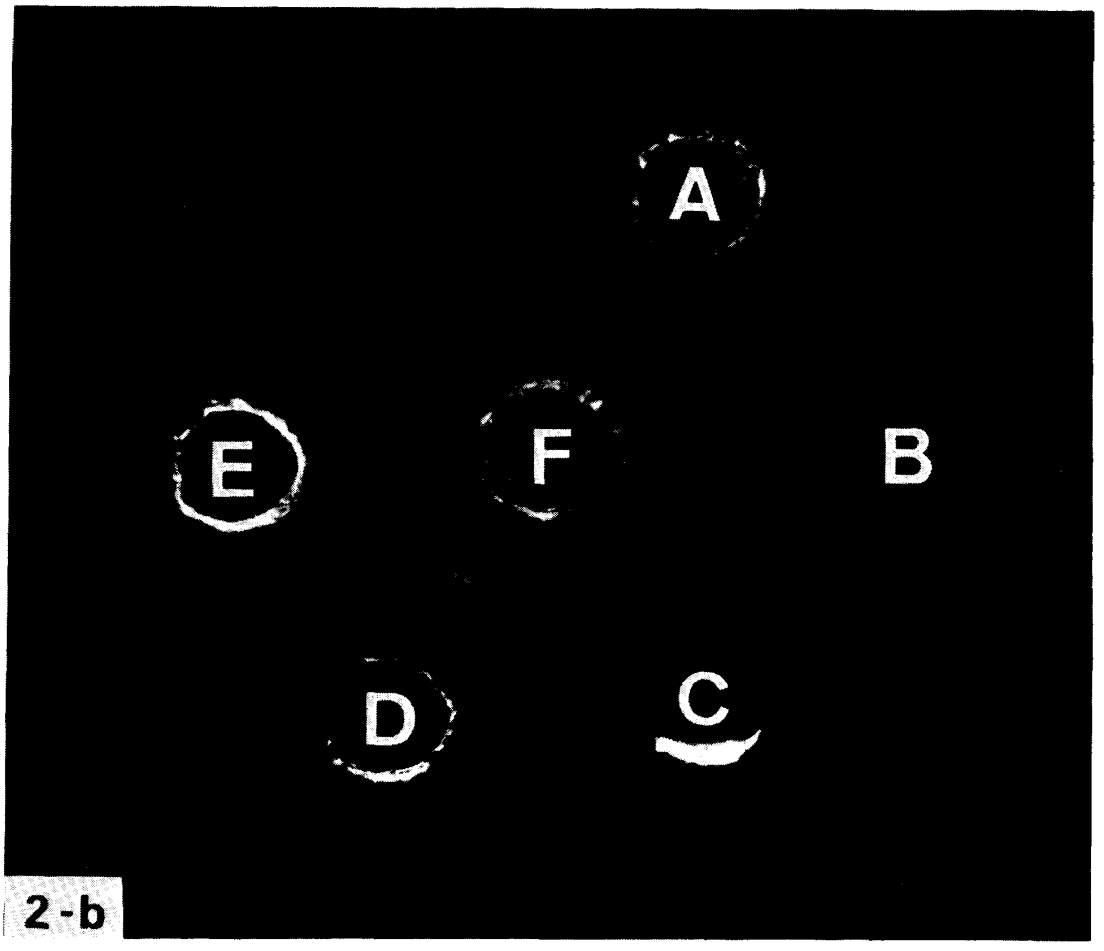

Fig. 2-(b). Ouchterlony test of HMDBP. (A): Anti-mouse IgG antiserum, (B): anti-mouse IgA antiserum, (C): anti-mouse IgM antiserum, (D): anti-mouse serum antibody, (E): anti-mouse HMDBP (160,000) antiserum, (F): 10 μg of HMDBP.

DNA-binding proteins were chromatographed on DEAE-cellulose, and high molecular weight glycoproteins were eluted with 10 mM phosphate buffer (pH 6.8) containing 0.12 M KCl (Fig. 1–b). In the DEAE-cellulose column eluted with 0.12 M KCl about 20 mg of protein was recovered from the 130 mg in the starting fraction. The 0.12 M KCl fraction was found to be contaminated with immunoglobulins. To separate HMDBP from the immunoglobulins, isoelectric focusing column chromatography was performed (Fig. 1–c), because the isoelectric point of HMDBP is at pH 4.8, while that of the immunoglobulins is at pH 6.0 or more. A pH 5.0 to 8.0 gradient was chosen because HMDBP becomes insoluble when the electric focusing voltage is raised from 400 to 1,000. This means that HMDBP is an acidic protein. The precipitates at pH 4.8 were purified on Sephadex G-200 gel chromatography to isolate HMDBP (Fig. 1–d). Recovery was about 3 mg of protein from 100 ml of ascites tumor fluid.

Immunochemical analysis Fig. 2–a shows the immunoelectrophoresis patterns of mouse serum and HMDBP on agarose gel. The precipitation line between HMDBP and anti-mouse serum antibodies is visualized as a line at the β-region of mouse serum. Fig. 2–b shows precipitation lines in agarose by the double diffusion test. Distinct precipitation lines are visible between HMDBP and its antibodies and between HMDBP and anti-mouse serum antibodies, the precipitation lines being fused with each other. No precipitation line is demonstrated between

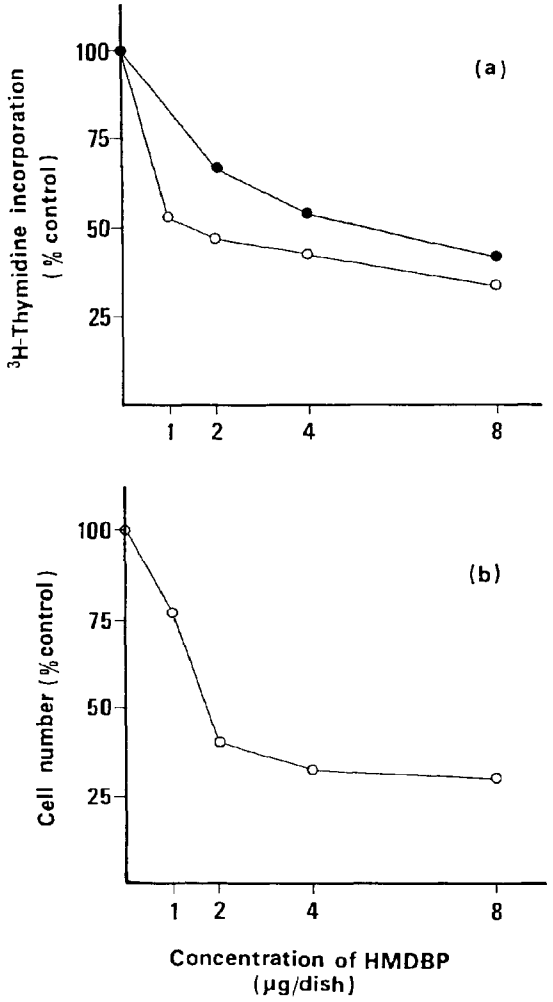

Fig. 3. Tumor cell growth in culture media with and without HMDBP. Meth-A fibrosarcoma (○——○) and (●——●) were assayed in DEM containing 0.3% FCS. (a): incorporation of ^{3}H-thymidine, and (b): number of tumor cells.

HMDBP and antibodies against mouse immunoglobulin subclasses.

Suppression by HMDBP of tumor cell growth in vitro Fig. 3 shows the results of in vitro experiments. Meth-A fibrosarcoma cells in control dishes increased to about 1.7 times the original number during the period from the medium change to the end of culture when DEM was supplemented with 0.3% FCS. The proliferation of tumor cells was suppressed by 70%, when 8.0 μg of HMDBP was adeed (Fig. 3–a and –b). The HMDBP obtained from Meth-A ascitic fluid of BALB/c mice suppressed the isogeneic tumor as described above, but this protein also suppressed the growth of xenogeneic tumor cells from HeLa S3. These tumor cells increased to 1.6 times the number at the start of culture in DEM containing 0.3% FCS. Incorporation of ^{3}H-thymidine into HeLa S3 cells was suppressed to neally the same degree as incorporation of ^{3}H-thymidine into Meth-A fibrosarcoma cultivated in culture media supplemented with 0.3% FCS containing HMDBP (Fig. 3–a).

DISCUSSION

A number of studies on DNA-binding proteins have been reported. These were found in the serum and body fluids of several animal species[2], in human cerebrospinal fluid[7], in rat Yoshida ascitic fluid[8] and recently in the serum of patients with malignant diseases[3~5]. These DNA-binding proteins were reported to migrated from α- to β-regions on immunoelectrophoresis. Degradation products of C3[4] and of fibronectin[5] are examples of β-region components. In contrast, α-component involves several immunosuppressive factors such as α-fetoprotein[16] and immunoregulatory α_2-globulin[17].

In the present experiments, DNA-binding proteins were obtained from the ascitic fluid of Meth-A fibrosarcoma-bearing mice, and the main fraction had a molecular weight of 160,000 daltons. The fraction was separated and purified on DEAE-cellulose column and by isoelectric focusing. Purified HMDBP is an acidic glycoprotein migrating on immunoelectrophoresis to the β-globulin region. The main protein (160,000) in HMDBP has not more than two subunits covalently bound by disulfide bonds, so it is different from the nine coplement components and immunoglobulin subclasses. Interestingly enough, it can suppress the growth of syngeneic Meth-A fibrosarcoma and of xenogeneic HeLa S3 in vitro. Three DNA-binding proteins have been obtained from rat Yoshida sarcoma ascitic fluid which also appear to be able to suppress tumor cell growth; their molecular weights are 200,000 or more, 150,000 (IgG), and 100,000 or less daltons[8]. From the serum of patients with malignant disease, two DNA-binding proteins with molecular weights of 170,000 and 140,000 daltons have also been isolated[6]. Therefore, HMDBP is unique among the DNA-binding proteins.

Recently attention has been focused on the control of cell growth by proteins in serum or exudates. First, Krøll et al.[8] described some of the proteins which can suppress tumor cell growth in vitro. Next, α-globulin from normal human serum was shown to inhibit the growth of mitogen-stimulated lymphoid cells[18] and tumor cells[9]. A soluble factor from tumor cells, the molecular weight of which was 140,000 daltons, inhibited the multiplication of various cells in vitro[19]. The contact-inhibitory culture medium in which melanoma cells had grown inhibited the growth of various tumor cells, and the active principle was found to have a molecular weight of

160,000 daltons[10]. The HMDBP obtained from Meth-A fibrosarcoma ascitic fluid is a partially purified fraction of DNA-binding proteins, and the active principle may be 160,000 daltons. This suppressed the growth of mouse Meth-A fibrosarcoma cells and of human HeLa S3 cells in vitro. The tumor suppressing activity of HMDBP has been shown in the present experiments. It seemes to inhibited the growth of various types of tumor cells. The active principle in HMDBP needs to be purified further. Although the mode of action is not yet known, HMDBP, as found in serum and/or exudates but not in subcutaneous solid tumors (data not shown), appear to be able to counteract certain tumor growth-stimulating factors.

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SUMMARY

A high molecular weight DNA-binding protein (HMDBP), from the ascitic fluid of mice with Meth-A ascites, was partially purified with isoelectric focusing column chromatography. The HMDBP was shown to be acidic in isoelectrophoresis. It did not cross-react with IgA, IgM and IgG. The major HMDBP was determined, by sodium dodecylsulfate gel electrophoresis, to be about 160,000 daltons. In tumor cell cultures, HMDBP suppressed the growth of tumor cells. Therefore, this high molecular weight DNA-binding β-globulin may play a role in controlling tumor growth.

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